Commentary

Comment on 'On the logic of the application of double-inhibitor titrations for the elucidation of the mechanism of energy coupling' by P.S. O'Shea and M. Thelen (FEBS Letters 176 (1984) 79–82)

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In the paper 'On the logic of the application of double-inhibitor titrations for the elucidation of the mechanism of energy coupling', FEBS Lett. 176 (1984) 79–82, O'Shea and Thelen criticise the use of so-called double-inhibitor titrations to discriminate between chemiosmotic and non-chemiosmotic mechanisms of energy transduction. The kind of experiments described are in fact uncoupler titrations in the presence or absence of an inhibitor of the secondary proton pump, as introduced by Baum et al. [1] and further applied by Hitchens and Kell [2] and others [3,4].

On page 82 of their paper the authors argue that for titrations of ATP synthesis with uncoupler, in the presence of an inhibitor of the ATP synthase (the secondary proton pump) the experimentally obtained curve c (see fig.1) can be equally well explained by chemiosmotic as by non-chemiosmotic behaviour. This conclusion conflicts with earlier explanations in which curve c was reversed for non-chemiosmotic behaviour whereas curve b was predicted for chemiosmotic behaviour [2–4].

In this comment we aim to show that the arguments of O'Shea and Thelen are incorrect. Following the description of O'Shea and Thelen we start with a modified form of their eqn 1, on page 81:

$$n_o J_o = (L_u + L_p)\Delta p \qquad (1)$$

In this equation the Δp generating flow is given by the rate of electron transport (J_o) times the number of protons translocated over the membrane per oxygen atom consumed (n_o), while the Δp dissipating flow is a linear function of Δp and the leakage terms (L_u and L_p). In this description the natural leak of the membrane is neglected because this factor is relatively small compared with the two other proton dissipating processes. These other two pro-

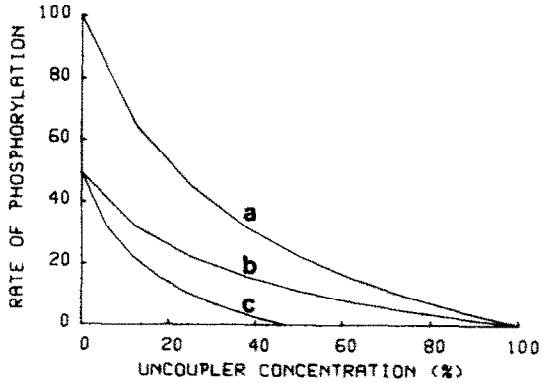

Fig.1. A schematic representation of a typical double-inhibitor titration. This figure was taken from the paper of P.S. O'Shea and M. Thelen in FEBS Lett. 176 (1984) 79–82. Curve a represents an uncoupler titration without inhibition of ATP synthase, curve c a titration in the presence of an ATP synthase inhibitor. Curve b indicates the behaviour expected according to the interpretations of Hitchens and Kell [2] in the case of chemiosmotic behaviour. The form of curves a and c is taken from the data of [2] (see fig.2 in [2]).

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cesses are leakage of protons through ATP synthase molecules and leakage of protons over the membrane mediated by uncoupler. L_p represents the proportionality constant that indicates how ATP synthesis depends on Δp, L_u the proportionality constant that indicates how uncoupler-induced proton leaks across the membrane depend on $\Delta p L_p$ can be considered proportional to the concentration of ATP synthase molecules in the membrane, whereas L_u varies proportionally with the concentration of uncoupler.

According to the chemiosmotic theory the rate of ATP synthesis (J_p) is determined by the Δp and by the concentration of active ATP synthase molecules (L_p), i.e. $n_p J_p = L_p \Delta p$ (at constant ΔG_p). N_p is the amount of protons translocated over the membrane per mole of ATP synthesized. However, in the treatment given by O'Shea and Thelen the dependency of ATP synthesis on Δp is neglected. This leads them to conclude on page 82: "The J_p term may thus be considered to be equivalent to the L_p term" and, in the next paragraph: "One would expect, therefore, that the L_u term would have to increase by a much smaller amount to compete with the much depressed L_p term". To us this statement is against the principles of chemiosmosis. Increase of the L_u term decreases the Δp and has no effect on L_p. We would argue: when a certain percentage of the ATP synthase molecules is inhibited, Δp remains constant or will increase a little (cf. [5]), since less protons can flow across the membrane via the ATP synthase molecules. Thus, not less but rather the same amount (or perhaps more) uncoupler is needed to lower Δp, and thus ATP synthesis, to the same relative extent, compared with the uninhibited case. Or, in terms of O'Shea and Thelen, L_u will have to increase by a *larger* amount to obtain the same decrease in ATP synthesis performed by the active ATP synthase molecules, compared with the control.

The argument given above can also be developed in a more quantitative way. Following the treatment of O'Shea and Thelen, we assume that the force-flow relationships given in eqn 1 are linear. Eqn 1 can be mimicked in this case by the electric circuit given in fig.2. In this circuit V is identical to Δp, R_u ($= 1/L_u$) represents the resistance experienced by protons flowing across the membrane catalyzed by an uncoupler, i_u the rate at which protons are flowing through R_u, R_p ($= 1/L_p$) the resistance experienced by protons flowing through the ATP synthase molecules and i_p the proton flow through ATP synthase molecules.

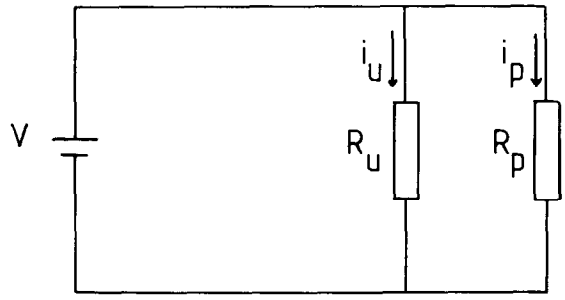

Fig.2. Electric circuit representation of an energy-transducing membrane involved in oxidative phosphorylation. Details are given in the text.

Using the electric circuit model, we compare the slopes at zero uncoupler concentration of the titration curves in fig.1. In the treatment we give below, Δp is assumed to remain constant (cf. [5]) upon inhibition of part of the ATP synthase molecules. In fig.1 it can be seen that the slope of curve c is the same as for curve a or is even steeper. This was experimentally found for oxidative phosphorylation in chromatophores [2] and for ATP-energized reverse electron transfer at site 1 in submitochondrial particles [3,4]. In the case of curve b, however, the slope is less steep compared with curve a. A quantitative expression is now needed for the slope and the dependence of the slope on the amount of active ATP synthase molecules (L_p). In fig.1, the slope can be represented by:

$$d(J_p)/d(\text{uncoupler concentration}).$$

In the electric circuit of fig.2 the analogous expression is $d(i_p)/d(L_u)$. In order to differentiate $d(i_p)$ with respect to $d(L_u)$ we derive from fig.2 (according to Ohm's law: $V = i_p R_p = i_u R_u$): $i_p = L_p i_u / L_u$, which leads to eqn 2.

$$d(i_p)/d(L_u) = -L_p i_u / L_u^2 \qquad (2)$$

From eqn 2 it follows directly that the slope is proportional to L_p. Upon inhibition of part of the ATP synthase molecules, L_p will decrease, which implies that the slope will be less steep. From the treatment given here it also can be concluded that curve b would be expected and not curve c.

Some results of inhibitor-uncoupler titrations performed in our laboratory show that it is indeed possible to obtain a titration plot as represented by curve b (fig.1) in a system that is expected to behave in a chemiosmotic way. We performed these experiments with liposomes containing bacteriorhodopsin and ATP synthase and obtained a titration plot corresponding to curve b upon partial inhibition of the ATP synthase molecules with 8-azido-ATP, as shown in fig.3. More studies with this co-reconstituted system will be presented elsewhere.

From the above considerations we conclude that O'Shea and Thelen do not make it 'abundantly clear' that there is also a possibility to predict curve c with the chemiosmotic model. Also, the experiment presented in fig.3 shows that in the case of expected chemiosmotic behaviour curve b is in fact obtained. From fig.3B it can be seen that the normalised curves are identical. This indicates that the slopes are proportional to L_p. Taken together with previous results of uncoupler-inhibitor titrations [2–4] we conclude that the objections of O'Shea and Thelen do not invalidate the concept that this type of experiment can discriminate between a straightforward chemiosmotic mechanism and other coupling mechanisms.

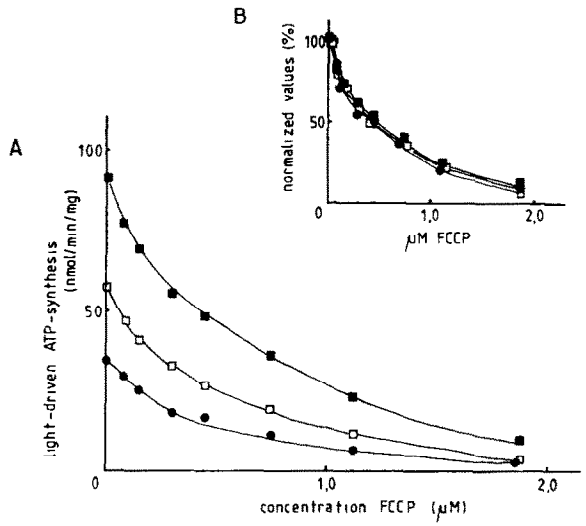

Fig.3. Uncoupler titration of ATP synthesis measured in liposomes containing bacteriorhodopsin and ATP synthase, prepared according to the sonication gel filtration method as described by Van der Bend et al. (see [6]). Inhibition of ATP synthase was obtained by illumination at 350 nm of liposomes in the presence of 200 μM 8-azido-ATP for different periods (■, 0 min; □, 10 min; ●, 20 min). Residual 8-azido-ATP was afterwards removed by gel filtration. ATP synthesis was measured as described by Van der Bend et al. [6]. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was used as uncoupler. In panel A absolute activities are plotted, in panel B relative activities.

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